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Oral and nasal microbiota in Parkinson's disease

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ABSTRACT

Introduction: Parkinson's disease (PD) is associated with neuropathological changes in olfactory and gastrointestinal tissues, and PD patients frequently suffer from hyposmia, hyposalivation, and dysphagia. Since hyposmia and gastrointestinal dysfunction are frequently premotor symptoms, it has been speculated that an external, for example microbial, agent could trigger the pathologic process in the corresponding organs, subsequently spreading to the central nervous system. We recently showed evidence for compositional differences between the fecal microbiota of PD patients and control subjects. In this study, our objective was to explore a possible connection between nasal and oral microbiota and PD.
Methods: We compared the oral and nasal bacterial communities of PD patients (oral: n = 72, nasal: n = 69) and control subjects (oral: n = 76, nasal: n = 67) using a 16S rRNA gene amplicon sequencing approach.
Results: Oral and nasal microbiota differed markedly from each other, with no notable similarity within subjects. Oral microbiota of PD patients and control subjects had differences in beta diversity and abundances of individual bacterial taxa. An increase in the abundance of opportunistic oral pathogens was detected in males, both with and without PD. Our data did not reveal convincing differences between the nasal microbiota of control subjects and PD patients.
Conclusion: The oral microbiome deserves additional research regarding its connection to PD and its biomarker potential. The higher abundance of oral pathogens in men underlines the importance of monitoring and promoting male dental health.

1. Introduction

Besides motor symptoms, Parkinson's disease (PD) is associated with a broad spectrum of non-motor symptoms (NMS) that may precede motor symptoms and severely affect patients' quality of life. One major aspect of these disturbances is gastrointestinal (GI) dysfunction [1]. This symptomatology has been linked to alpha-synuclein related neuropathology in the enteric nervous system which shows a rostro-caudal gradient with particularly eminent changes in the salivary glands and the lower esophagus [2]. Recently, comparable changes were found in mucosal sensory nerve terminals of the oropharynx, larynx, and upper esophagus [3]. Correspondingly, decreased salivation, notwithstanding the common phenomenon of drooling, and dysphagia are frequently seen in PD patients [1]. Another early NMS is hyposmia, which is associated with neuropathological changes in the olfactory system, and may be present in over 90% of PD patients, often preceding motor symptoms [4]. The early involvement of olfactory and GI structures in PD could be compatible with an exogenous factor acting in the upper respiratory and GI tracts, possibly initiating the neurodegenerative cascade leading eventually to PD [5]. A microbial aetiology has been discussed in this context, considering the continuous exposure to external microbes and the thin epithelium in the nose and enteric organs [6].

Various studies have suggested a potential role for oral microbiota in human disease, such as diabetes and cardiovascular disease [7]. Periodontal diseases are also associated with PD [8]. Based on our promising findings regarding gut microbiota in PD [9] and the strong involvement of olfactory and perioral tissues in early PD, we carried out a corresponding analysis on nasal and oral samples from

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subjects who had been included in our gut microbiome study.

2. Materials and methods

The study was approved by the ethics committee of the Hospital District of Helsinki and Uusimaa, and all participants gave informed consent. The study was registered at clinicaltrials.gov (NCT01536769).

2.1. Study subjects and sampling

Initially, 152 subjects (76 PD patients and 76 control subjects with no signs of parkinsonism, frequency-matched for age and gender) were included in the study. Details on recruitment, exclusion criteria and clinical data collection have been published previously [9]. Additionally, we excluded subjects with any known active chronic disease of the nasal or oral cavity, nasal sinuses, salivary glands, or pharynx (with the exception of mild allergic or nonspecific rhinitis). Periodontal disease or dental caries were not excluded and patients did not undergo a systematic clinical assessment of the nasal and oral cavity or dental status. We excluded patients with a history of major ear, nose or throat (ENT) surgery (with the following exceptions: enrolment was allowed 6 months after antrostomia, septal surgery, or surgery for obstructive sleep apnea) and those that reported one or more of the following in the preceding 2 months: inflammation of oral or perioral tissues, a major dental procedure, more than twice-weekly use of glucocorticoids (nasal, inhaled, oral, or parenteral) or intranasal anticongestive drugs. None of the subjects had used antibiotics within the last month nor smoked within the past 6 months.

Nasal and oral samples were obtained with cotton swabs during clinic visits. For nasal samples, single use sterile nasal specula were used for insertion of the swab to avoid contact with the nares. The swab was inserted into the nasal passage between the septum and middle turbinate aiming towards the upper part of the nasal cavity. Oral samples were acquired by gently brushing the buccal and sublingual mucosa bilaterally with the swab. We chose to sample from these locations due to their close proximity to the orifices of the submandibular and parotid ducts. Immediately after sampling, swabs tips were placed into small aliquot containers and in ice. Within 20 min, samples were transferred to −80 °C for storage.

2.2. Sequencing and sequence analysis

The V1-V3 regions of the 16S rRNA gene were amplified using universal bacterial primers and sequenced with an Illumina MiSeq sequencer. Full methodological details are provided in the Supplement.

Due to technical reasons, the numbers of subjects included in the final oral and nasal sample sets differed: the oral data set consisted of 72 PD patients and 76 control subjects, while the nasal data set had 69 PD patients and 67 control subjects. The total amount of raw reads for the oral data set was 21 645 150, and for the nasal data set 8 638 162. Raw sequence data have been uploaded to the European Nucleotide Archive (accession no. PRJEB14536).

The sequence analysis, including OTU (Operational Taxonomic Unit) clustering and taxonomy assignment, was done in mothur [10]. Oligotyping of nasal *Staphylococcus* sequences was done using the oligotyping pipeline as instructed by the authors [11]. Full details are provided in the Supplement.

2.3. Statistics

Statistical analyses were performed using the R statistical programming language. All *p*-values are double-tailed, with statistical significance accepted at an alpha of 0.05. Non-rarefied data without singletons were used for calculating Shannon and inverse Simpson alpha diversity indices, which were compared statistically using Kruskal-Wallis rank sum tests. Beta diversity, based on Bray-Curtis dissimilarity, was visualized with Non-Metric Multidimensional Scaling (NMDS) and compared statistically with adonis (an implementation of PERMANOVA) from the vegan package [12]. Differential abundance analyses were done with DESeq2 [13], which uses Generalised Linear Models with a negative binomial distribution, with Benjamini-Hochberg method for multiple comparison correction. Full details are provided in the Supplement.

3. Results

3.1. Clinical data

The groups were generally similar regarding clinical parameters (Table 1): gender, age and BMI did not differ significantly between the control and PD groups. As expected, the PD patients had worse scores for NMS measurements, such as dysphagia assessed by the Swallowing Disturbance Questionnaire (SDQ) [14], drooling assessed by the Sialorrhea Clinical Scale for PD (SCS-PD) as a surrogate of saliva volume [15], and hyposmia assessed by the Sniffin' Sticks 16-item smell identification test [16]. A history of cholecystectomy was more common in the PD group, and this difference was statistically significant in the nasal data set ($p = 0.017$), but not in the larger oral dataset (p=0.073). All but two PD patients were using antiparkinsonian medications. Control subjects used more frequently warfarin and statins than the PD patients, and more commonly had a history of atrial fibrillation or TIA/ischemic stroke.

3.2. Alpha and beta diversity

The overall alpha diversity (a sample-specific estimation of diversity that quantifies both "species" richness and evenness), measured with the Shannon and inverse Simpson indices, was higher in the oral than the nasal data (Kruskal-Wallis rank sum test: $p = 2.12^{*}10^{-10}$). Statistical comparisons of alpha diversity within each data set did not suggest any differences between the control and PD groups (Kruskal-Wallis rank sum test: $p > 0.67$ for both data sets; Fig. S1).

Regarding beta diversity (which quantifies community composition similarity between samples), an ordination plot of Bray-Curtis dissimilarity visualizing both data sets implied that the oral and nasal bacterial communities were very distinct, with non-overlapping clustering by sample location (Fig. 1). Oral and nasal samples from the same individual did not appear more similar than those of unrelated subjects (Wilcoxon rank sum test: $p = 0.88$ for dissimilarity of oral and nasal microbiota in related vs. unrelated pairs of samples; Fig. S2).

Beta diversity comparisons with adonis for the oral data suggested a minor but statistically significant community difference between PD patients and control subjects (adonis: $p = 0.0138$, $R^2 = 0.0113$) and a borderline difference for gender (adonis: $p = 0.0494$, $R^2 = 0.0098$). For the nasal data, there were no statistically significant community differences (data not shown).

3.3. Oral microbiota

The oral data set was composed of 7996 non-singleton OTUs, distributed among 129 genera, 76 families, 41 classes, 23 orders, and 15 phyla. The most common genera were *Streptococcus, Haemophilus, Neisseria, Veillonella* and *Prevotella* (Fig. 2A, Table S1).

Out of the variables used in GLMs (isolation date, gender, age at

Table 1
Clinical data.

	Subjects in oral comparisons			Subjects in nasal comparisons		
	control (n = 76)	PD (n = 72)	*p*-value	control (n = 67)	PD (n = 69)	*p*-value
Female sex (%)	50	51.39	0.871	50.75	49.28	1
Age (years, mean ± SD)	64.33 ± 6.95	65.36 ± 5.50	0.317	64.43 ± 6.80	65.25 ± 5.56	0.447
BMI (median, IQR)	26.23 [23.74–28.11]	25.99 [23.72–29.3]	0.946	26.25 [23.89–28.16]	26.25 [24.09–29.16]	0.94
Alcohol units per week (median, IQR)	2 [0–6]	1 [0–5]	0.399	2 [0–5.5]	1 [0–6]	0.796
SDQ total score (median, IQR)	0 [0–1.25]	4 [1.75–8]	<0.001	0 [0–2]	3 [1–8]	<0.001
SCS-PD total score (median, IQR)	0 [0-0]	2 [1–6]	<0.001	0 [0-0]	2 [1–6]	<0.001
Sniffin' Sticks score (median, IQR)	13 [12–14]	7 [6–9]	<0.001	13 [12–14]	7 [6–9]	<0.001
Atrial fibrillation (%)	17.11	4.17	0.016	19.4	4.35	0.008
TIA/ischemic stroke (%)	35.53	5.56	<0.001	37.31	5.8	<0.001
non-major ENT surgery (%)	21.05	22.22	1	20.9	23.19	0.837
Cholecystectomy (%)	3.95	12.5	0.073	1.49	13.04	0.017
Levodopa (%)	0	52.78	<0.001	0	52.17	<0.001
COMT inhibitor (%)	0	13.89	<0.001	0	15.94	<0.001
Dopamine agonist (%)	0	77.78	<0.001	0	79.71	<0.001
MAO inhibitor (%)	0	68.06	<0.001	0	71.01	<0.001
Anticholinergic (%)	0	8.33	0.012	0	8.7	0.028
Warfarin (%)	14.47	1.39	0.005	16.42	1.45	0.002
Statins (%)	53.95	20.83	<0.001	53.73	18.84	<0.001
UPDRS-III; mean ± SD		31.9 ± 8.9			31.8 ± 8.7	
UPDRS total (I-IV); mean ± SD		45.9 ± 13.4			46.0 ± 13.4	
Years from motor onset; median [IQR]		5 [3–8]			5 [3–8]	
Years from NMS onset; median [IQR]		6 [2.5–10.5]			7 [2.75–10.25]	
Hoehn and Yahr stage; n (%)						
1		4 (5.6%)			3 (4.3%)	
1.5		2 (2.8%)			2 (2.9%)	
2		23 (31.9%)			23 (33.3%)	
2.5		27 (37.5%)			27 (39.1%)	
3		16 (22.2%)			14 (20.3%)	

PD: Parkinson's disease; SD: Standard Deviation; IQR: interquartile range; BMI: body mass index; SDQ: Swallowing Disturbance Questionnaire [14]; SCS-PD: Sialorrhea Clinical Scale for PD [15], Sniffin' Sticks: Sniffin' Sticks 16-item smell identification score [16], TIA: transient ischemic attack, ENT: ear, nose and throat, COMT: catechol-*O*-methyl transferase, MAO: monoamine oxidase, UPDRS: unified Parkinson's disease rating scale [17], NMS: non-motor symptoms.

oral sample collection, history of non-major ENT surgery, alcohol units per week, SDQ total, SCS-PD total, and control vs PD), control versus PD was associated with the largest number of differentially abundant taxa: 11 families, 10 genera and 25 OTUs (Table 2). Many of the taxa differentially abundant between PD patients and control subjects (Fig. 2B, Fig. S3, Fig. S4, Table S2) contain potential opportunistic oral pathogens: *Prevotella*, Prevotellaceae, *Veillonella*, *Solobacterium*, Veillonellaceae, Lactobacillaceae, and Coriobacteriaceae increased in PD, while *Capnocytophaga*, *Rothia*, *Kingella*, *Leptotrichia*, *Actinomyces*, and Leptotrichiaceae decreased. Other differentially abundant taxa were *Haemophilus*, *Neisseria*, *Gemella*, *Corynebacterium*, *Granulicatella*, an unclassified Flavobacteriaceae OTU, Pasteurellaceae, Neisseriaceae, Micrococcaceae, Carnobacteriaceae, and Corynebacteriaceae, all found decreased in the PD group, while *Moryella* and Erysipelotrichaceae were increased.

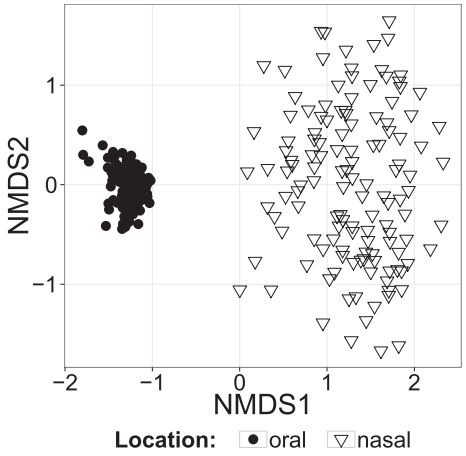

Fig. 1. Non-metric multidimensional scaling (NMDS) ordination plot of the oral and nasal samples. Ordination based on Bray-Curtis dissimilarity calculated with genus-level data, subsampled to 2000 reads per sample. Each point represents one sample; the closer the points are to one another, the more similar the microbiome compositions of the samples.

Regarding other clinical variables, we found evidence for increased abundances of taxa containing potential opportunistic oral pathogens in males relative to females (with or without PD): *Lactobacillus*, *Capnocytophaga*, *Leptotrichia*, *Veillonella*, *Aggregatibacter*, *Porphyromonas*, and *Prevotella* (Table S3). Age was positively correlated with *Porphyromonas* Otu000029 abundance and with an unclassified Prevotellaceae OTU, and negatively correlated with the abundance of Flavobacteriaceae. One taxon with a statistically significant differential abundance was discovered for each of the other model variables, but further investigation suggested them to be the result of statistical noise, with the possible exception of *Gemella* Otu000162, which was positively correlated with a history of non-major ENT surgery.

3.4. Nasal microbiota

The 2212 non-singleton OTUs of the nasal data represented 553 genera, 177 families, 96 orders, 49 classes, and 28 phyla. The dominant genus in both subject groups was *Corynebacterium*,

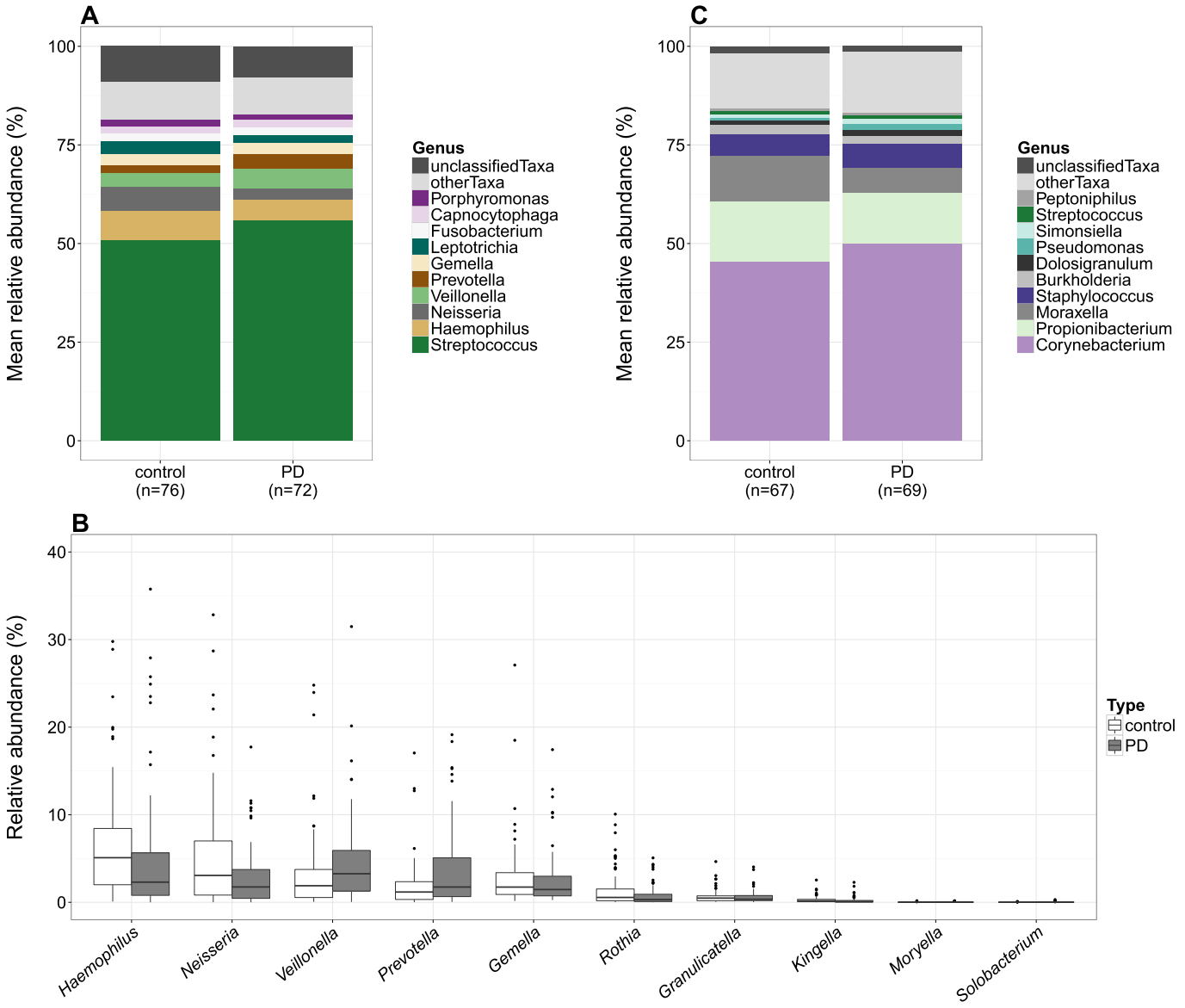

Fig. 2. Relative abundances of bacterial genera in the control and Parkinson's disease (PD) groups. A. 10 most common genera in the oral data, B. Differentially abundant genera (DESeq2 $p < 0.05$) in the oral data. Lower and upper hinge of the box: 1st and 3rd quartile; whiskers: 1.5 * interquartile range; line: median. C. 10 most common genera in the nasal data.

followed by the genera *Propionibacterium, Moraxella, Staphylococcus* and *Burkholderia* (Fig. 2C, Table S4).

For differential abundance comparisons, the model included DNA extraction batch, gender, age at sample collection, history of non-major ENT surgery, Sniffin' Sticks score, and control vs PD. A single taxon was differentially abundant between the control and PD groups on each of the taxonomic levels explored: Otu00006 (*Staphylococcus*), the genus *Marmoricola*, and the family Flavobacteriaceae (Fig. S5, Table S5). To further explore the potentially interesting *Staphylococcus* OTU, we performed oligotyping, which resulted in three oligotypes, none of which appeared differentially abundant between control subjects and PD patients (Fig. S6; Supplementary results).

Considering the other variables in the model, DNA extraction batch and sex were associated with the largest number of differentially abundant taxa, while age at sampling, history of ENT surgery and the Sniffin' Sticks score were associated with a handful of taxa each (Table 2, Table S6). Out of the taxa differentially abundant for batch or sex, 63 were shared between the two variables, and all but two of these were more abundant in the second batch and in female subjects (Fig. S7).

4. Discussion

The oral and nasal microbial communities were very different from one another, with no overlap in the most common taxa, a higher alpha diversity in the oral data, and distinctive communities when comparing beta diversity. These results are consistent with previous studies where oropharyngeal microbiota were found to be more diverse than nasal microbiota, and more similar in a specific anatomical site across individuals than within a specific individual [18].

Table 2
Number of differentially abundant (DESeq2 $p < 0.05$) taxa per variable and taxonomic level. Only variables with at least one differentially abundant taxon are shown. SCS-PD: Sialorrhea Clinical Scale for PD [15]; ENT: ear, nose and throat; SDQ: Swallowing Disturbance Questionnaire [14].

Variable	Family	Genus	OTU
Oral			
control vs PD	11	10	25
sex	2	10	16
age at sample collection	1	0	2
SCS-PD total	1	0	0
history of ENT surgery	0	0	1
SDQ total	0	0	1
Nasal			
DNA extraction batch	41	59	70
sex	24	19	53
age at sample collection	0	1	9
history of ENT surgery	4	3	2
Sniffin' Sticks	0	0	4
control vs PD	1	1	1

4.1. Oral microbiota

The oral microbial composition inferred from our study is consistent with previous findings [19,20]. *Streptococcus* seems to dominate the oral communities in virtually all studies, and potential oral pathogens are widely distributed among subjects, including orally healthy individuals, supporting the idea that their pathogenicity is of an opportunistic nature [19].

All members of the Prevotellaceae family that were differentially abundant between control subjects and PD patients in the oral cavity were increased in PD, as opposed to our findings regarding gut microbiota, which showed a reduction in members of this family in PD cases [9]. This is not contradictory, given that different species and/or strains can be present in the gut and oral cavity. The *Prevotella* genus includes known opportunistic oral pathogens [21], and it is possible that their increase in PD is associated with hygiene differences, such as motor or non-motor symptoms leading to a reduction in the efficiency of oral care. Lower frequencies of tooth brushing, less visits to a dentist, as well as an increased prevalence of caries, periodontal diseases, and tooth loss have been linked to PD [8]. On the other hand, a recent study reported good self-assessed dental health care in PD patients [22]. It should also be noted that in our study, many of the taxa that contain bacteria associated with oral pathologies were reduced in abundance in PD (seven out of fourteen OTUs, two out of five genera, and one out of five families). This means that although oral hygiene may play a partial role, it cannot alone explain the overall differences. Another potential factor influencing the oral microbiota could be diet. For example, gut microbiome studies suggest that *Prevotella* grows preferentially on fibre- and carbohydrate-rich diets [23]. PD patients have been reported to consume more vegetable-derived proteins and carbohydrates [24], which supports our findings for oral *Prevotella*. However, other studies have suggested that diet does not significantly affect oral microbial communities [25]. Overall, at this point, the reasons for the observed difference in *Prevotella* abundance remain unclear and it is premature to decide whether the detected differences have clinical relevance for PD or whether some of the taxa could be exploited as biomarkers.

All other taxa with statistically significant differential abundances between PD patients and control subjects were found to be reduced in the PD group, except for *Moryella* and Erysipelotrichaceae, which were increased. However, further investigation suggests that the statistical significance associated with the latter two taxa is the result of statistical noise. To our knowledge, none of these groups are recognised as including opportunistic oral pathogens except for Flavobacteriaceae (e.g. *Capnocytophaga*). Why these taxa are reduced in abundance in PD is a question that remains open.

The finding of higher abundances of various genera and families that contain opportunistic oral pathogens in males, regardless of disease status, supports studies that indicate worse oral hygiene in males [26]. This could explain the generalised abundance increase in these organisms in our study, and may also emphasize the need to closely monitor dental health, particularly in male PD patients.

Age was positively correlated with *Porphyromonas* Otu000029 abundance and with the unclassified Prevotellaceae Otu000438, and negatively correlated with the abundance of the Flavobacteriaceae family. The genus *Porphyromonas* is associated with periodontal disease (e.g. *Porphyromonas gingivalis*), as are the Prevotellaceae and Flavobacteriaceae families, the latter through *Capnocytophaga*, which contains various species that are part of the normal oral microbiome and have pathogenic potential. Visualisation of the data suggests that both the Prevotellaceae OTU and the Flavobacteriaceae family may be false positives, leaving only the increase in the *Porphyromonas* OTU reliably associated with increasing age. It is possible that this OTU is involved in an increase in periodontal diseases with increasing age.

4.2. Nasal microbiota

The most common genera in our nasal microme samples were similar to those reported in previous studies [18,27]. Our data did not offer convincing support for altered nasal microbiota in PD patients: we did not detect any differences in alpha or beta diversity between the control and patient groups. Out of the three taxa that appeared differentially abundant between control subjects and PD patients, two were less abundant in PD patients: the genus *Marmoricola* and the family Flavobacteriaceae. *Marmoricola* has only been isolated from environmental sources [28]. Several genera of the family Flavobacteriaceae include human pathogens, but for them to be of particular interest, the differential abundance should be detectable for specific genera or OTUs. Both taxa could well be false positives. A *Staphylococcus* OTU was more abundant in PD patients, but further exploration with oligotyping did not support this difference. It should be mentioned that the mucosal area closest to the olfactory bulb, the olfactory epithelium, cannot be reached from the nostrils with non-invasive methods and that the nasal microbiome may show regional differences. So we cannot exclude that PD related nasal microbiome alterations could have been detected if samples had been acquired from the olfactory epithelium. However, more invasive procedures were beyond the scope of this exploratory study.

The most prominent differences between nasal samples were based on DNA extraction batch and sex. The nasal samples were handled in the order of arrival at the laboratory, which corresponded to the sampling date. This means that seasonal variation could be one explanation for the differences, but since the samples collected in the same season were in the same DNA extraction batch, it is difficult to tell apart technical and seasonal effects. A particularly puzzling finding are the 63 taxa that were differentially abundant in relation to both batch and sex, with a higher abundance in females and in the second of two batches. A recent study found a difference in bacterial densities in the nares of male and female subjects, but not in any specific taxa [29]. One explanation for our finding could be that a lower density of bacteria and thus a lower biomass in female subjects' samples might make reagent contaminants more prominent. Similar phenomena related to contamination and the amount of template DNA in PCR have been reported previously [30].

5. Conclusions

Our results support previous studies indicating that oral bacterial communities are more diverse than nasal communities. It also suggests that the oral microbiota of PD patients differ from those of control subjects as assessed through beta diversity and differential abundance analyses. Differences were also detected between sexes, with a higher abundance of taxa that include opportunistic oral pathogens in males, which is supported by previous studies correlating gender and hygiene practices. We did not find any indications for differences in nasal microbiota between control subjects and PD patients.

The exploratory nature of this study means that any attempts to explain the mechanisms behind the connection between PD and oral microbiota would be highly speculative, and further research is needed to gain a better understanding. At this point, we can neither tell whether oral microbiota are connected to the aetiology of PD, nor whether the observed changes are a consequence of host changes associated with the disease or the result of external factors. Regardless of the underlying mechanisms, the detected differences support further exploration of oral microbiota as a possible biomarker for PD.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.parkreldis.2017.02.026.

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